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ABSTRACT

Title of Thesis: Stress Biomarkers in a Rat Model of Decompression Sickness

James A. Caviness Jr., Master of Science, 2005

Thesis directed by: Elizabeth Montcalm-Smith, Adjunct Assistant Professor, Department of Military and Emergency Medicine

INTRODUCTION: Immune, stress, and inflammatory reactions may contribute to decompression sickness (DCS). Using ELISA and PCR, this research determines whether levels of genomic/proteomic responses in 7 stress sensitive markers are associated with DCS.

MATERIALS AND METHODS: Rats undergoing a test dive (175 fsw/60 min, rapid decompression) were observed for DCS signs. Animals exercised on a rotating cage (~3 m/min) throughout the dive and subsequent 30 min observation period. Rats suffering DCS and those not were euthanized: blood/tissues were collected for ELISA and PCR analysis.

RESULTS: Lung Egr-1 showed statistical significance between bent and not-bent animals.

CONCLUSIONS: Lung Egr-1 is elevated with DCS in this model. Further research is warranted to determine what role Egr-1 genes play in DCS. Studies could investigate pulmonary DCS, evaluate Egr-1 as a marker for individual DCS susceptibility, or point toward targets for non-recompressive interventions.

STRESS BIOMARKERS

IN A RAT MODEL OF

DECOMPRESSION SICKNESS

by

James A. Caviness Jr.

Thesis submitted to the Faculty of the
Military and Emergency Medicine Applied Human Biology Graduate Program
Uniformed Services University of the Health Sciences
In partial fulfillment of the requirements for the degree of
Master of Science 2005

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Stress Biomarkers in a Rat Model of Decompression Sickness

Statement of Objectives:

The objectives of this research are (1) determine, in a rodent model, the effect of decompression on immediate/early stress response genes and known acute stress biomarkers and, and (2) if there is any correlation to DCS outcome or susceptibility with these stress response genes or stress biomarkers.

Statement of Hypotheses and Specific Research Aims:

The research hypothesis is that rats that develop DCS during a dive manifest a greater stress response resulting in greater amounts of early stress response genes or proteins compared to those rats that do not develop DCS. The null hypothesis is that there is no difference in the amount of early response genes or proteins between rats developing DCS and those rats that do not develop DCS.

The Specific Aims of this investigation are to:

(1)Determine if manifestation of decompression sickness is accompanied by genomic and proteomic responses in, p-selectin, endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), endothelin, heat shock protein 27 (HSP27), heat shock protein 70 (HSP70), hemoxygenase 1 (HO-1), c-reactive protein (CRP), and early growth response gene (Egr-1).

(2) To determine if specific alterations in gene expression and/or product proteins of #1 serve as predictors for DCS occurrence or susceptibility.

Introduction and Background

Decompression sickness (DCS) occurs when divers who have incurred an excess inert gas load under hyperbaric conditions, ascend to the surface (decompress) too rapidly allowing inert gas bubbles to form in the blood and tissues. Tissues typically affected include joints, skin, lungs, and central nervous system. Severity can range from trivial to fatal.

A review of the literature reveals no controlled study of early/immediate stress response genes or biomarkers of stress in the setting of DCS. Researchers have instead focused on specific plausible mediators of injury in DCS (1, 2, 3, 4, 5). However, attempts to identify the mechanism for DCS have yielded surprisingly little concrete information. A literature review identified candidate stress response genes or proteins that are produced immediately in response to acute stress within 3 general categories:

1) modifiers of vascular function

2) early non-specific stress response genes

3) classic hypothalamic-pituitary-adrenal (HPA) stress response

Recently, Martin and Thom's elimination of DCS in rats by blocking neutrophil adhesion is a step toward identifying the mechanism behind DCS (6). However, the mechanism by which DCS triggers neutrophil adhesion remains unexplored. In other disease models, P-selectin has been demonstrated to play a crucial role in neutrophil adhesion and activation (7, 8, 9). Endothelin, a potent vasoconstrictor, has been shown to increase in response to endothelial shear stress in rats (11, 12), and to also play a role in stress-induced hypertension (13, 14). Other research has shown Nitric Oxide (NO) plays

a critical role in inhibiting the rolling and adhesion of neutrophils to the endothelium (10). In addition to its effect on neutrophils, Nitric Oxide is a potent vasodilator. NO has not been studied in respect to hyperbaric stress and will be addressed in the following section due to its relationship with the non-specific stress response Heat Shock Protein (HSP) genes.

Nitric Oxide levels are driven by HSP. Numerous researchers have demonstrated the stimulation of HSP 27 and HSP 70/72, by a variety of stressors, to include exercise (15, 16, 17, 18, 19, 20). Ischemic stress to rat spinal cords resulted in a significant rise in HSP 27 and HSP 70 (21). While rarely specifically investigated, the oxidative stress present in the hyperbaric/hyperoxic environment of diving is likely another stimulus of HSP production. One study has identified elevated levels of HSP70 in rats during hyperbaric conditions (22). Another has revealed that hydrostatic pressure alone causes elevated HSP70 and HSP90b production (23, 24). HSP70 results in the induction of iNOS in macrophages and increased NO production; whereas HSP60 has the same function in monocytes (25). HSP90 binds nNOS and eNOS and stimulates NO production (26) HSP70 protects rats from gastric mucosal damage via NO (27) and the resistance of August rats, as compared to Wister rats, to MI has been shown to be due to August rats having higher activity of HSP70 and a resultant higher NO (1.8 times higher) (28). In a landmark series of experiments, Wisloff and Brubakk demonstrated that both aerobic exercise and NO have protective effects against DCS (29, 30, 31). NO has been shown to have a protective effect to ST changes on treadmill testing (32). While there is no clear relationship to hyperbaric or decompression stress, hemoxygenase has been shown be have a rapid and early response to ischemic stress (21, 37).

C-reactive protein is a well-known acute phase reactant that has been demonstrated to be elevated in oxidative stress disease states, as well as to modify NO release (33, 34).

The classic stress response includes the activation of the hypothalamic-pituitary-adrenal axis (HPA). This response occurs to a variety of stressors resulting in the rapid release of corticotropin releasing hormone (CRH) by the hypothalamus, inducing adrenocorticotropic hormone (ACTH) release by the pituitary, which in turn induces glucocorticoid production by the adrenal gland. The net result is a rapid production of epinephrine, glucose, and corticosteroid. This response holds true for rats. The individual rat response to stress dictates the level and extent of HPA activation (31). Researchers have recently started to characterize the genetic machinery responsible for the HPA response. For epinephrine, transcription factor Egr-1 is required for the epinephrine synthesizing enzyme phenylethanolamine N-methyltransferase (PNMT). Egr-1 dramatically up regulates in response to stress in the spine (21), the adrenal medulla (36), and brain (37).

This proposal will examine plausible early-immediate stress responses in rats exposed to DCS in 3 major categories:

1) Vascular modifiers: p-selectin mRNA, nitric oxide: eNOS mRNA and iNOS mRNA, endothelin

2) Non-specific stress response: HSP: HSP27 mRNA, HSP70 mRNA, hemoxygenase-1 mRNA, and c-reactive protein

3) Classic HPA axis: Egr-1 mRNA

The broad aims of the proposed research are twofold:

(1) To determine, in a rodent DCS model, whether changes in levels of genomic and biomarkers of stress response occur in p-selectin, eNOS mRNA, iNOS mRNA, endothelin, HSP27 mRNA, HSP70 mRNA, hemoxygenase 1 mRNA, c-reactive protein, and egr-1 mRNA.

(2) To determine if specific alterations in gene expression and/or product proteins of #1 serve as predictors for DCS occurrence or susceptibility.

Materials and Methods

Animals

Male Sprague-Dawley rats comprising the control groups in the ongoing Naval Medical Research Center (NMRC) protocol entitled, "Acclimation to Decompression Sickness (DCS) and its Mechanisms in Rats" were utilized. As the title suggests, the study investigates the effect of a previous hyperbaric exposure to a subsequent hyperbaric exposure/rapid decompression likely to produce DCS. The control rats utilized for my investigation, however, had no such pre-hyperbaric exposure.

Diving protocol

The NMRC protocol uses a dive profile estimated to have a 60% DCS rate. On the test dive day, rats undergo a 175FSW dive for 60 minutes. During the dive, they are in a rotating cylinder cage, ensuring all rats are equally active. They are then rapidly decompressed to the surface and observed for 30 minutes for signs of DCS. At the end of the observation period, they are euthanized and tissue and blood samples are taken.

Sample Selection

A total of 7 separate test dive's control rats were available for use. For each test dive, there were 5 control rats. Within each set of 5 control rats, pairs were selected so that RT-PCR was done comparing levels of the target gene products for a rat that had no evidence of DCS (annotated as lived), and a rat that had DCS (annotated as bent). Rats that died were not included as the time from death to tissue and blood sampling was highly variable, and this post-mortem period would undoubtedly result in degradation of RNA or altered levels in target genes.

A total of seven pairs of lived-versus-bent rats were matched from these 7 dives

In addition to serum, brain, liver and lung tissue were collected for analysis.

RNA preparation

Brain, liver, and lung tissue samples from rats in the control group of the NMRC DCS acclimation study were flash frozen in liquid nitrogen or placed in RNAlater and subsequently stored at minus 70 degrees Celsius. Tissue was cut into approximately 30mg sections, placed in 0.2ml ice-cold TRIZOL, and homogenized by ulrasonification. An additional 0.8 ml TRIZOL was added followed by 0.2 ml chloroform, and the samples were then shaken vigorously and set at room temp for 2 minutes. Samples were centrifuged at 10,800 rpm for 15 minutes at 4 degrees Celsius. Next, 0.5 ml of the supernatant was transferred to a new tube with 0.5 ml isopropyl alcohol, mixed by vortex, and then set at room temperature for 10 minutes. They were centrifuged at 10,800rpm for 10 minutes at 4 degrees Celsius. The supernatant was removed and discarded. 1 ml 75% ethanol was added and mixed by vortex. A final centrifuge was performed at 8480rpm for 5 minutes at 4 degrees Celsius. The pellet was dried briefly and then 13ml RNAse-free

water was added. RNA was quantified by spectroscopy and was deemed adequate if the A260/A280 ratio was 1.8 or greater.

For the first group of primers subjected to RT-PCR, the 7 pairs of rats were used. Upon preparation for RT-PCR of the second group of primers, the first two pairs were found to have RNA degradation as evidenced by absent *B*-actin bands.

RT-PCR

Relative mRNA levels were determined using gene-specific primer pairs from Invitrogen (Table 1). PCR reactions were carried out using 1 uL RNA sample, 1 uL each of sense and antisense primer, and 47 uL of Titanium 1-step PCR mix (BD Biosciences). PCR products were then subjected to gel electrophoresis with 2 uL loading buffer and 18 uL PCR mRNA. The resulting bands were photographed and analyzed on a SynGene MultiGenius Bioimaging System with SynGene GeneSnap software.

Table 1
RT-PCR Primers

EGR-1	Sense	CCCGTATGCTTGCCCTGTTGAGTC
	Antisense	CCCGTTGAGGTGCTGAAGGAGTTG
ENOS	Sense	TACGGAGCAGCAAATCCAC
	Antisense	CAGGCTGCAGTCCTTTGAT
HO-1	Sense	ACTTTCAGAAGGGTCAGGTGTCC
	Antisense	TTGAGCAGGAAGGCGGTCTTAG
HSP27	Sense	GGTTTCCCGATGAGTGGTCTC
	Antisense	CTCCGCTGATTGTGTGACTGC
HSP70	Sense	GAGTCCTACGCCTTCAATATGAAG
	Antisense	CATCAAGAGTCTGTCTCTAGCCAA
INOS	Sense	AAATGCAGGAGATGGTCCGC
	Antisense	GTCTTGTGCCTTTGGGCTCC
P-Selectin	Sense	TGTATCCAGCCTCTTGGGCATTCC
	Antisense	TGGGACACGAAGTGATGTTACACC

ELISA

Blood samples of the rats was centrifuged and the white cell fraction frozen at minus 70 degrees Celsius. Samples thawed on ice were examined for levels of endothelin and c-reactive protein per the manufacturer's recommended protocols, Assay Designs' rat Big Endothelin ELISA and BD Biosciences' Rat C-reactive protein (CRP) ELISA, respectively. For ELISA, samples were run in duplicate. An automatic plate washer was used. Microtiter plates were read on Bio-Rad Laboratories Microplate Manager Model 680.

Statistical analysis

Both the SynGene MultiGenius GeneSnap System and BioRad Laboratories Microplate Manager report results into Microsoft Excel.

Statistical Analysis was done utilizing Windows Office XP Excel's Statistical Analysis Tools using the descriptive statistics and t-test: two-sample assuming equal variances. P value was set at 0.05 and the difference between groups was set as expected to equal zero (no difference).

RESULTS

A total of 7 sets of control rats were available for use. For each set, there were 5 control rats. Within each set of 5 control rats, pairs were selected so that RT-PCR was done comparing levels of the target gene products for a rat that had no evidence of DCS and a rat that had DCS. Rats that died were not included as the time from death to tissue and blood sampling was highly variable, and this post-mortem period would undoubtedly result in degradation of RNA or altered levels in target genes.

For each set of primers run on RT-PCR, the values from the SynGene GeneSnap load into Microsoft Excel. Values for each tissue biomarker level were divided by their respective *B*-actin level. This allows for a semi-quantitative comparison of the tissues from DCS rats (annotated as "bent") to the tissues of those rats without DCS (annotated as "lived").

The primers were ordered in two groups. The 1st group comprised HO-1, HSP27, HSP70, and P-selectin. The gel was run so that for each biomarker, the lived (no DCS) and bent (DCS) samples for each tissue system were adjacent. Using Microsoft Excel, statistical analysis was done using 2-sample t-test with equal variances. The null hypothesis was defined as: there is no difference in the values of the "lived" versus the "bent" rats. All values for each biomarker were analyzed and each tissue type was compared. Table 2 enumerates the results of analysis. None of the gene targets showed statistical significance. Subgroup analysis by type of tissue revealed that the lung demonstrated the lowest p-values except in the case of hemoxygenase. Hemoxygenase had a lower overall p-value than the individual tissue p-values. This suggests that a greater sample size might reveal a significant difference. A general trend of greater variability within the lived rats is noticed when compared to the bent rats. In most tissue systems, the variance of the lived rats was considerably greater than the bent rats. In total, there was simply a great amount of variability in the rats' stress response patterns. Figures 1-4 depict graphically the levels of target gene to *B*-actin ratio between the lived and bent rats. Although 7 pairs of rats were tested, the first two pairs had markedly elevated levels and were excluded from graphing to facilitate visual distinction among the other pairs. All seven pairs were included for analysis as presented in Table 2.

Table 2
RT-PCR RESULTS

	n	Mean	Standard Error	Standard Deviation	Sample Variance	P(T<=t) one-tail lived vs. bent
HO-1 lived	21	2.253848	1.109546	5.08458	25.85296	0.109746
HO-1 bent	21	0.826727	0.278824	1.277733	1.632601	
HO-1 Brain lived	7	2.700573	2.659234	7.035673	49.5007	0.237447
HO-1 Brain bent	7	0.683857	0.6346	1.678994	2.819021	
HO-1 Liver lived	7	2.158887	1.702233	4.503684	20.28317	0.237282
HO-1 Liver bent	7	0.879309	0.326685	0.864327	0.747062	
HO-1 Lung lived	7	1.902084	1.512092	4.000619	16.00495	0.27439
HO-1 Lung bent	7	0.917016	0.512608	1.356234	1.839371	
HSP27 lived	21	6.627975	5.594991	25.63947	657.3824	0.162045
HSP27 bent	21	1.030452	0.3594	1.646976	2.712531	
HSP27 Brain lived	7	1.233583	0.780767	2.065716	4.267182	0.387981
HSP27 Brain bent	7	0.948911	0.588961	1.558245	2.428128	
HSP27 Liver lived	7	0.544633	0.223624	0.591654	0.350055	0.266498
HSP27 Liver bent	7	1.140612	0.901096	2.384076	5.683817	
HSP27 Lung lived	7	18.10571	16.71543	44.22486	1955.838	0.163244
HSP27 Lung bent	7	1.001832	0.359951	0.952342	0.906955	
HSP70 lived	21	2.036915	1.208005	5.535774	30.6448	0.159719
HSP70 bent	21	0.7978	0.22666	1.038688	1.078872	
HSP70 brain lived	7	1.58453	0.981426	2.59661	6.742383	0.208799
HSP70 Brain bent	7	0.705983	0.363288	0.961171	0.92385	
HSP70 Liver lived	7	0.569116	0.271876	0.719315	0.517415	0.251034
HSP70 Liver bent	7	0.996708	0.554817	1.467907	2.154752	

HSP70 Lung lived	7	3.957098	3.542308	9.372067	87.83564	0.187908
HSP70 Lung bent	7	0.690711	0.253057	0.669526	0.448265	
P-selectin lived	21	3.934182	3.022178	13.84936	191.8048	0.135269
P-selectin bent	21	0.553269	0.152172	0.697338	0.48628	
P-selectin Brain lived	7	1.026658	0.813846	2.153235	4.636419	0.205486
P-selectin Brain bent	7	0.329715	0.083772	0.22164	0.049124	
P-selectin Liver lived	7	0.543783	0.235856	0.624016	0.389396	0.309894
P-selectin Liver bent	7	0.801121	0.446871	1.18231	1.397857	
P-selectin Lung lived	7	10.2321	8.98233	23.76501	564.7758	0.150642
P-selectin Lung bent	7	0.52897	0.078638	0.208058	0.043288	
EGR-1 lived	15	0.647528	0.145024	0.561674	0.315478	0.03164
EGR-1 bent	15	1.050915	0.14991	0.580599	0.337095	
EGR-1 Brain lived	5	0.963892	0.292422	0.653875	0.427552	0.216093
EGR-1 Brain bent	5	1.317347	0.311667	0.696909	0.485682	
EGR-1 Liver lived	5	0.71867	0.249723	0.558397	0.311807	0.347186
EGR-1 Liver bent	5	0.86475	0.257266	0.575264	0.330929	
EGR-1 Lung lived	5	0.260022	0.09558	0.213723	0.045677	0.007733
EGR-1 Lung bent	5	0.970646	0.211241	0.472349	0.223114	
eNOS lived	15	0.504836	0.115549	0.447521	0.200275	0.439274
eNOS bent	15	0.472964	0.171352	0.663644	0.440424	
eNOS Brain lived	5	0.500197	0.212475	0.475108	0.225728	0.28594
eNOS Brain bent	5	0.794074	0.451097	1.008684	1.017443	
eNOS Liver lived	5	0.557143	0.229083	0.512244	0.262394	0.341567
eNOS Liver bent	5	0.417939	0.23579	0.527241	0.277984	
eNOS Lung lived	5	0.457167	0.20325	0.454481	0.206553	0.139104
eNOS Lung bent	5	0.206879	0.070542	0.157737	0.024881	
iNOS lived	15	0.274892	0.0663	0.25678	0.065936	0.151405
iNOS bent	15	0.405524	0.105309	0.40786	0.16635	
iNOS Brain lived	5	0.278364	0.104541	0.233761	0.054644	0.343623
iNOS Brain bent	5	0.337156	0.094316	0.210897	0.044477	

iNOS Liver lived	5	0.178222	0.069032	0.154361	0.023827	0.383259
iNOS Liver bent	5	0.209963	0.076871	0.171888	0.029545	
iNOS Lung lived	5	0.36809	0.16109	0.360208	0.12975	0.183618
iNOS Lung bent	5	0.669452	0.271102	0.606202	0.367481	

Analysis is based on the ratio of each target gene's expression relative to the *B*-actin expression in the same sample tissue, as measured by the SynGene MultiGenius Bioimaging System and GeneSnap imaging software.

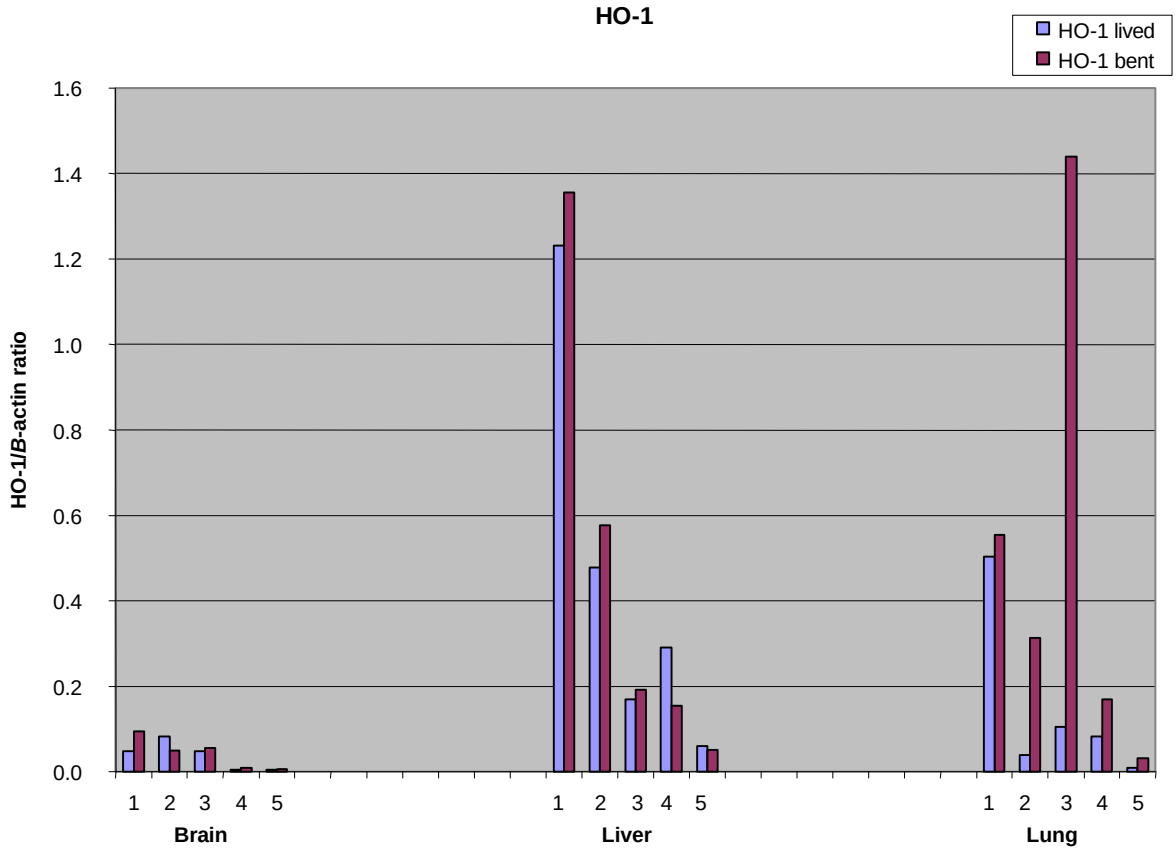

Fig 1. Results of RT-PCR followed by gel electrophoresis. GeneSnap imaging software used to quantify amount of hemoxygenase relative to *B*-actin in each tissue sample. Blue bars represent the HO-1/*B*-actin ratio in the rats that lived (did not have DCS), red bars represent the ratio in the rats that were bent (did have DCS). Numbers simply refer to the 5 lived-bent pairs

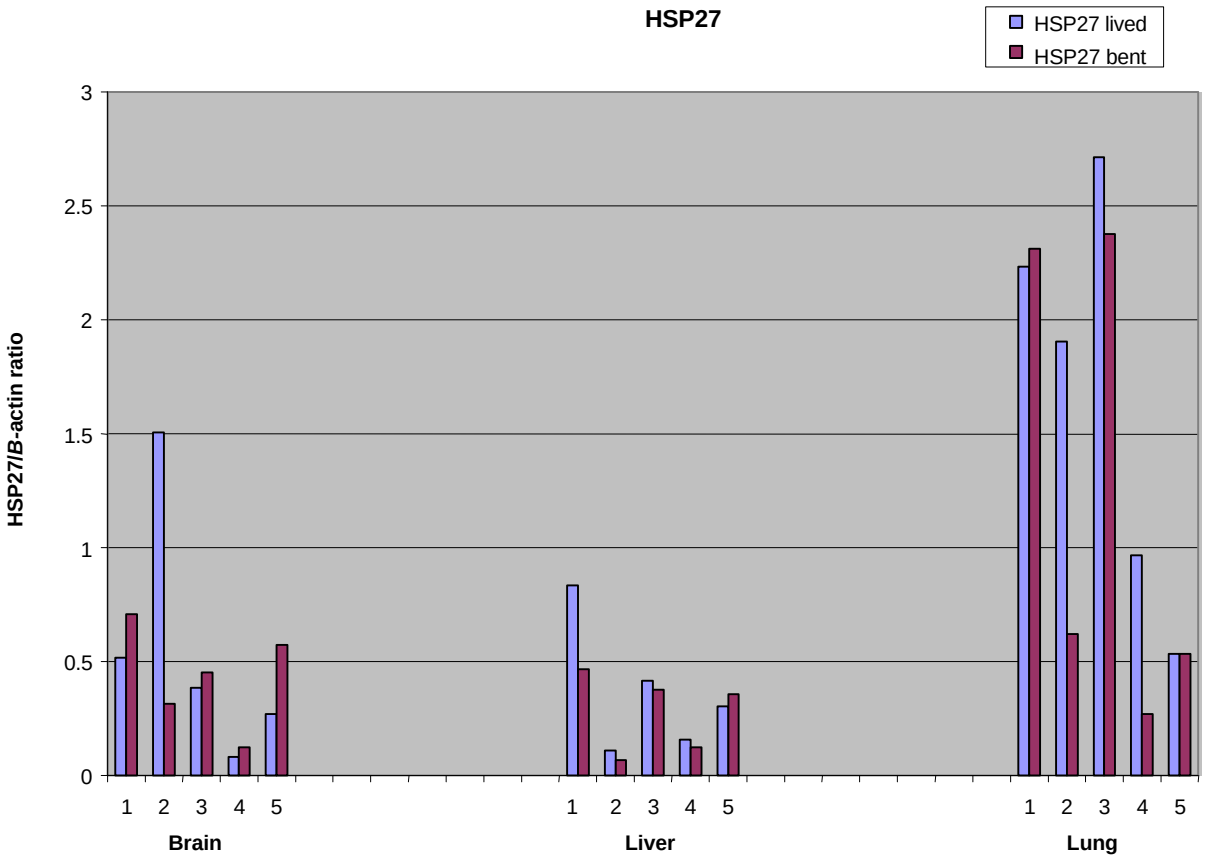

Fig 2. Results of RT-PCR followed by gel electrophoresis. GeneSnap imaging software used to quantify amount of heat shock protein 27 relative to *B*-actin in each tissue sample. Blue bars represent the HSP27/*B*-actin ratio in the rats that lived (did not have DCS), red bars represent the ratio in the rats that were bent (did have DCS). Numbers simply refer to the 5 lived-bent pairs

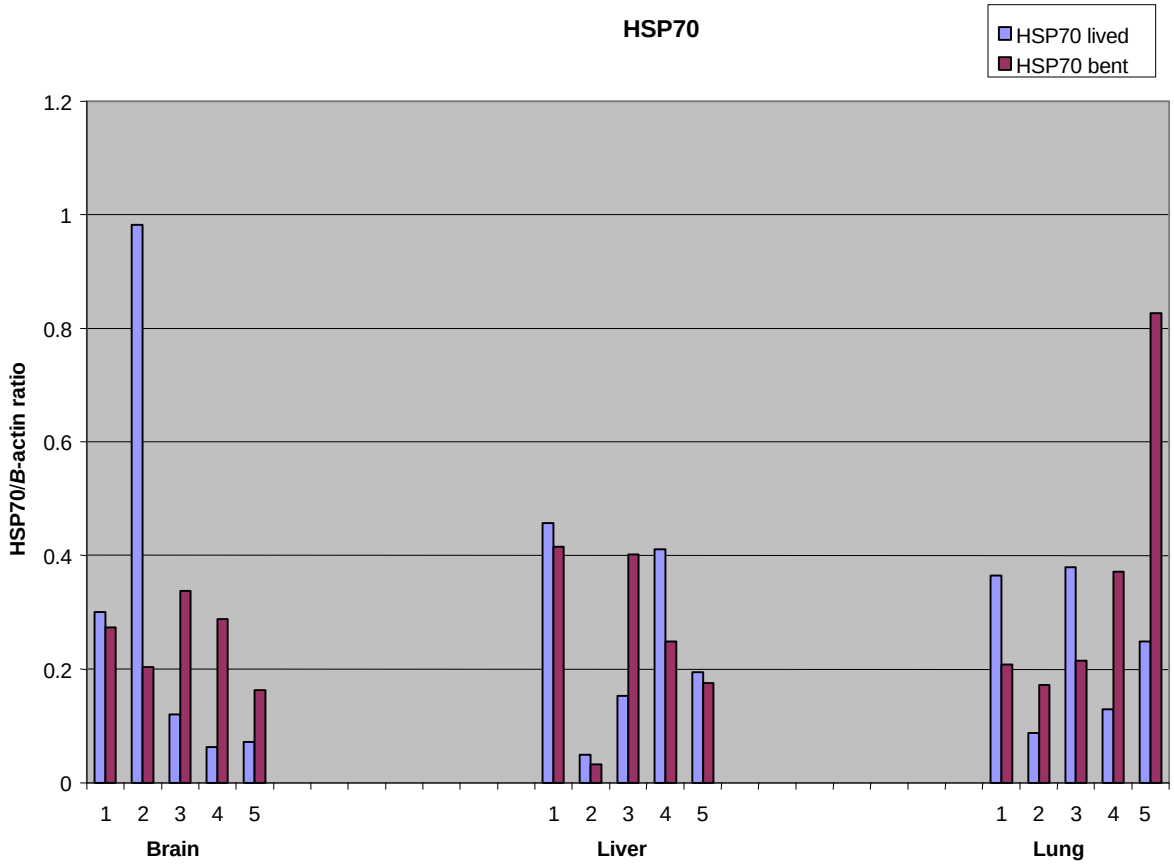

Fig 3. Results of RT-PCR followed by gel electrophoresis. GeneSnap imaging software used to quantify amount of heat shock protein 70 relative to *B*-actin in each tissue sample. Blue bars represent the HSP70/*B*-actin ratio in the rats that lived (did not have DCS), red bars represent the ratio in the rats that were bent (did have DCS). Numbers simply refer to the 5 lived-bent pairs

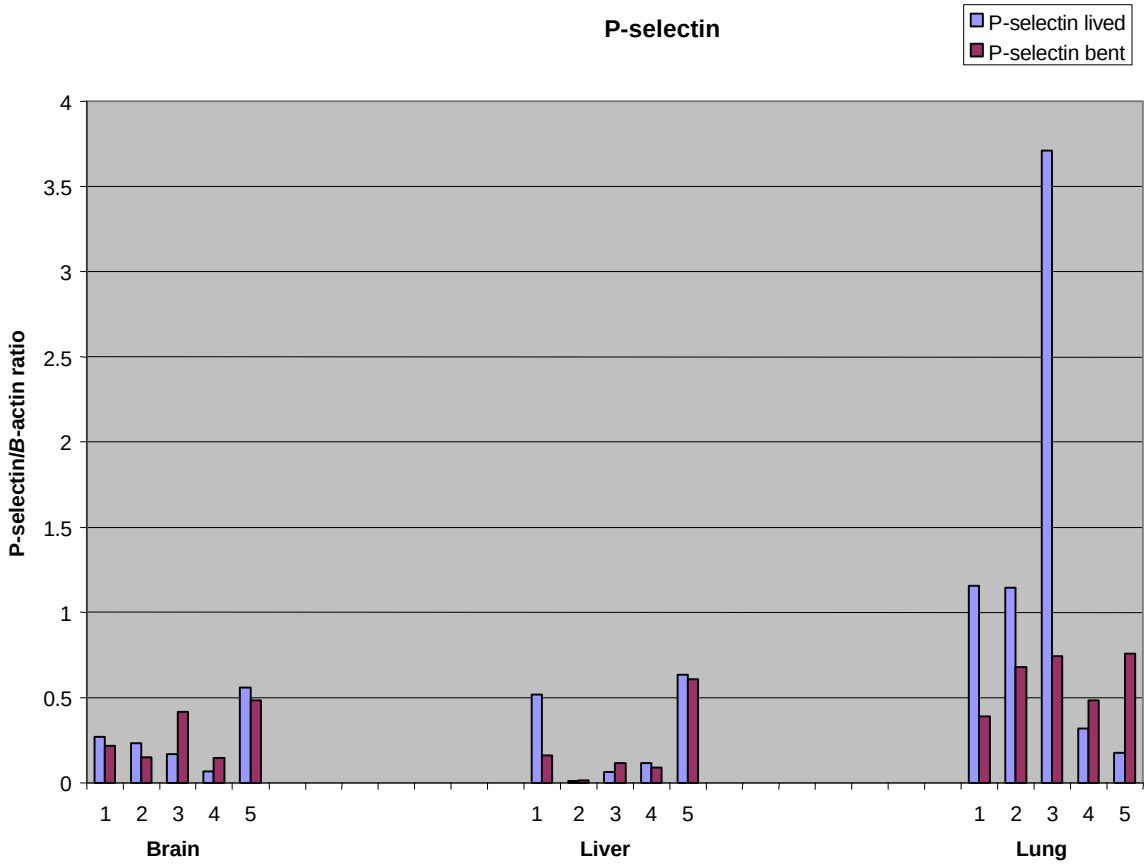

Fig 4. Results of RT-PCR followed by gel electrophoresis. GeneSnap imaging software used to quantify amount of P-selectin relative to *B*-actin in each tissue sample. Blue bars represent the p-sel/*B*-actin ratio in the rats that lived (did not have DCS), red bars represent the ratio in the rats that were bent (did have DCS). Numbers simply refer to the 5 lived-bent pairs

For the second group of primers, Egr-1, eNOS, and iNOS, only Egr-1 demonstrated statistical significance. Subgroup tissue analysis revealed that the lung was the source of statistical significance (p=.008). Results are enumerated in Table 2. In this second group of primers, variability was less dramatic. The same tissue samples were utilized, but the PCR mix was not from the same kit. A total of 5 titanium 1-step PCR kits were used. The first was used on the first two pairs of rats in the HO-1, HSP27, HSPO70, and P-selectin group, the next 2 kits were used to finish testing the remaining 5 rat-pairs for

these primers. The remaining 2 kits were used to test these same 5 rat-pairs on the Egr-1, eNOS, and iNOS primers.

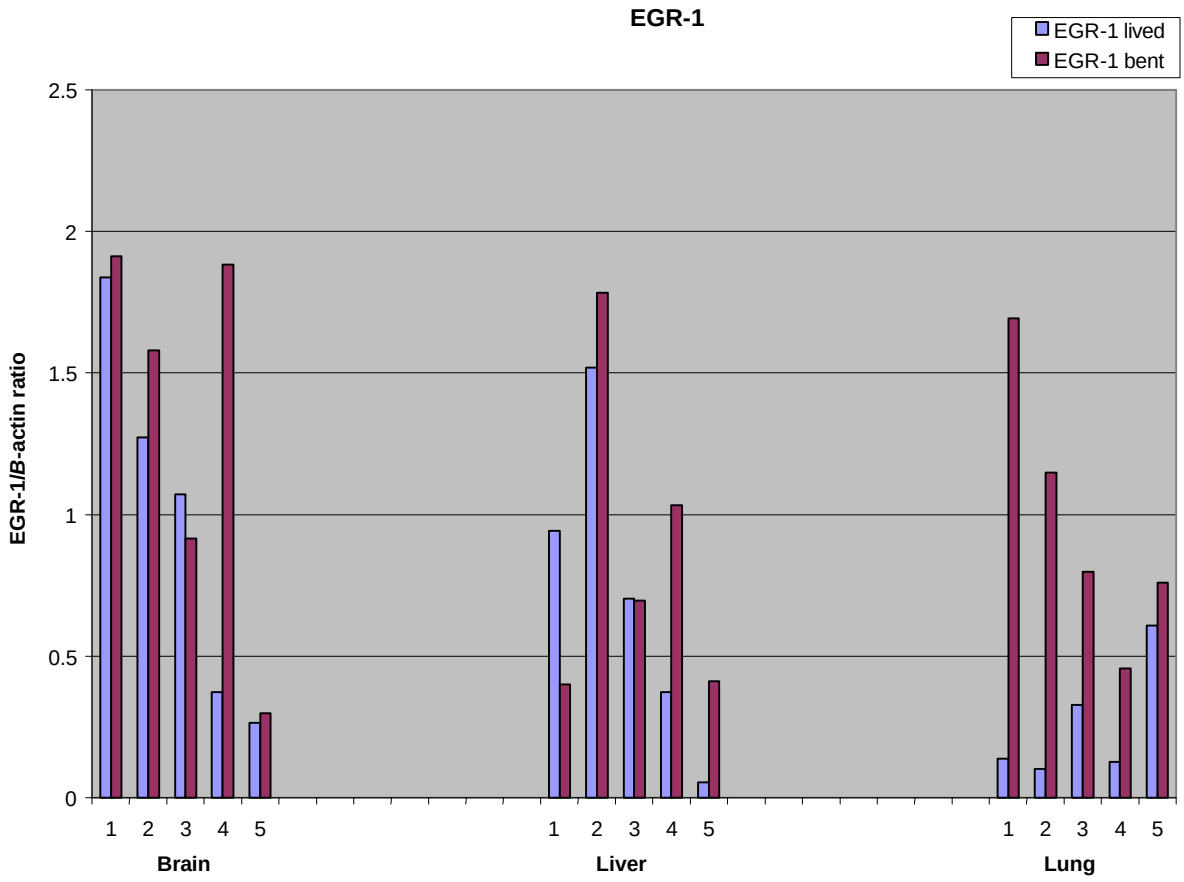

Fig 5. Results of RT-PCR followed by gel electrophoresis. GeneSnap imaging software used to quantify amount of Egr-1 relative to *B*-actin in each tissue sample. Blue bars represent the EGR-1/*B*-actin ratio in the rats that lived (did not have DCS), red bars represent the ratio in the rats that were bent (did have DCS). Numbers simply refer to the 5 lived-bent pairs

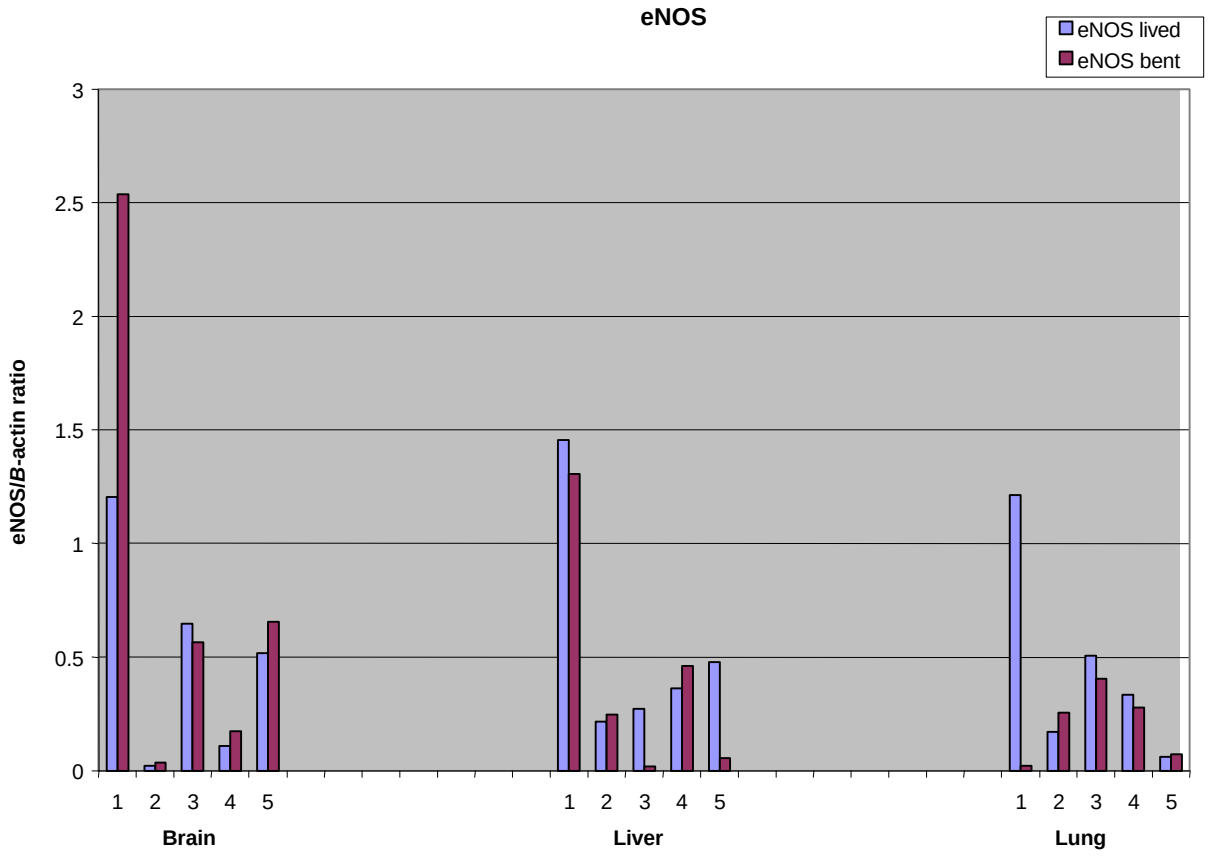

Fig 6. Results of RT-PCR followed by gel electrophoresis. GeneSnap imaging software used to quantify amount of endothelial nitric oxide synthase relative to *B*-actin in each tissue sample. Blue bars represent the eNOS/*B*-actin ratio in the rats that lived (did not have DCS), red bars represent the ratio in the rats that were bent (did have DCS). Numbers simply refer to the 5 lived-bent pairs

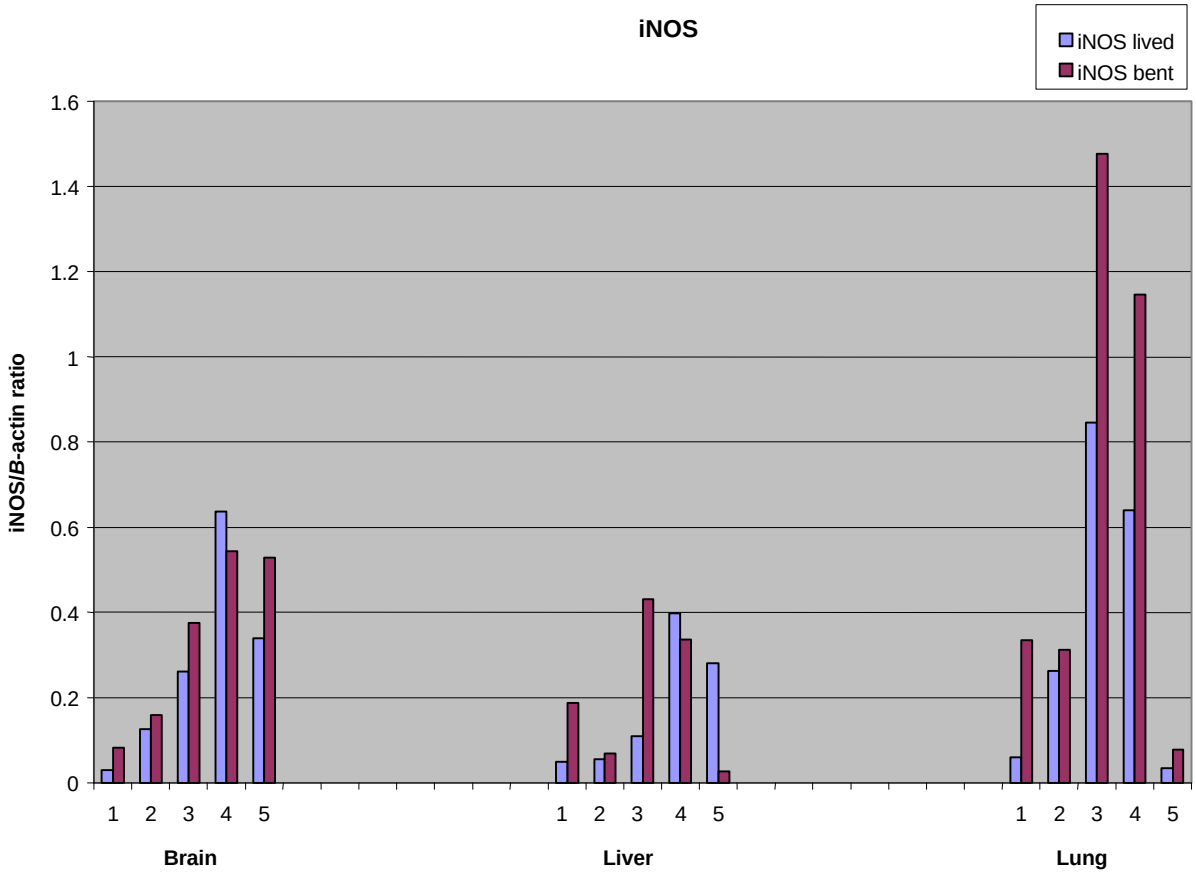

Fig 7. Results of RT-PCR followed by gel electrophoresis. GeneSnap imaging software used to quantify amount of inducible nitric oxide synthase relative to *B*-actin in each tissue sample. Blue bars represent the iNOS/*B*-actin ratio in the rats that lived (did not have DCS), red bars represent the ratio in the rats that were bent (did have DCS). Numbers simply refer to the 5 lived-bent pairs

Table 3
CRP

	n	Mean	Standard Error	Standard Deviation	Sample Variance	P(T<=t) one-tail
CRP lived	7	285.9891	29.1661	77.16626	5954.631	0.337203 lived vs. bent
CRP bent	11	298.8579	15.42378	51.15489	2616.823	0.398903 lived vs. died
CRP died	8	295.125	20.48963	57.95343	3358.599	0.441804 bent vs. died

CRP ELISA yielded a linear concentration curve with correlation coefficient of 0.867. A total of 26 serum samples were available for ELISA. Of these, 7 rats suffered no DCS, 11 suffered DCS, and 8 sustained severe DCS dying before the end of the

observation period. Two-sample t-test was done comparing no DCS to DCS, no DCS to dying, and DCS to Dying. As noted in table 3, none of these was statistically significant.

Endothelin ELISA had a textbook linear concentration curve with a correlation coefficient of .988. Unfortunately, following the manufacturer's suggested dilution of serum at 1:16 resulted in all values being below the lowest standard (0.78pg/mL). No additional kits were available to try different dilutions.

Discussion

The various studies of stress markers noted in the introduction have consistently shown a nearly two-fold or greater rise in the candidate genes used in this study. We did not see such dramatic changes between rats suffering DCS and those that did not. Clearly there is an important difference in this study: we are not investigating the change in genes or gene products pre- and post-stress, but rather the difference in levels between stressed rats with signs of DCS and stressed rats without signs of DCS. All rats underwent the same extreme stress of a 175 FSW dive for 60 minutes and then a rapid decompression to normal atmospheric pressure. Differences in the stress activated genes we investigated may not be dramatic enough between the rats suffering DCS and those rats not suffering DCS for us to detect. The stress of a hyperbaric exposure may be sufficiently unique from than other types of stressors previously studied, and thus a more subtle change in these biomarkers may be expected. Our study was not sufficiently powered to detect smaller differences in the stress biomarker response.

Equally plausible is the fact that DCS is not driven by any over or under-responsiveness of the early stress response genes. The strangeness of a hyperbaric chamber exposure, with the heating of compression, noise of compression and ventilation, the rapid cooling during decompression, and of course the generation of nitrogen bubbles throughout the body on rapid decompression may trigger a significant response in the genes we investigated in all rats, regardless of DCS, and that increasing numbers of rats would not have resulted in a different finding. As the stress response has not been well studied in DCS, it is not possible to say at this point if it plays a role in DCS or if a variation in stress response offers any protection or vulnerability to DCS. Despite the fact that DCS occurs rapidly, with the exception of Egr-1, the particular early response genes we examined may not play a role in its development.

Since recent work has pointed to white blood cells (6), it is possible that the best site for detecting early stress response biomarkers in DCS would be the serum itself. Stress response products such as HSP and NOS are known to act as cell signals to numerous sites including white blood cells. It is possible we did not detect them in the tissues we examined because they had been taken up elsewhere. Unfortunately, our design looked at only 2 serum markers and had no findings for CRP. The Endothelin ELISA showed levels below the sensitivity of the assay. We followed the manufacturer's directions for dilution of serum, and the lack of detectable endothelin indicates either that a lower dilution is needed to detect whatever endothelin may be activated in a DCS event, or that endothelin may simply not be released in detectable amounts in DCS.

There was a dramatic difference in the variability of results between the first group of primers (HO-1, HSP27, HSP70, and P–selectin) and the second group (Egr-1, eNOS,

iNOS). The procedure used was identical, but it is possible that there was degradation of primer or PCR kit components in the first group of primers run. A total of 5 kits of titanium 1-step PCR kits were used, with 3 being used for the first set of four primers, and the remaining 2 kits used for the second group of primers. Tissue samples were the same for both groups of primers used and it is possible that a freeze-thaw effect degraded RNA in the tissue samples; but, the PCR results of the second group of primers show good consistent *B*-actin bands and do not suggest that the RNA had degraded.

Egr-1 is one of the earliest responding genes to stress. Thus it is understandable that of all the stress response markers we looked at, the only one showing statistical significance was Egr-1. Absent the Lung, the Egr-1 findings were not much different than the other biomarkers. However, Egr-1 is significant in the lung to the point that it makes the results from the brain, liver and lung combined significant. This speaks to the fact that identifying not only the agent of interest but also finding the correct tissue (or serum) is critical to determining what is happening in DCS.

The lungs are the only route for safe release of nitrogen from the body during decompression. One of the most serious forms of DCS is called chokes. Chokes is an apt appellation for the clinical picture of DCS wherein the release of supersaturated nitrogen from the tissues results in a massive onslaught of bubbles that compromises the pulmonary vasculature, and subsequently respiratory and cardiovascular function. The increase in Egr-1 levels among rats that were stricken by DCS compared to those not suffering any DCS is dramatic. The mechanism for the dramatic response in Egr-1 warrants investigation. As the lungs are the route of nitrogen elimination on ascent form a dive and also the site of damage in pulmonary DCS, it is plausible that the local effects of nitrogen

bubbles on the lung parenchyma trigger the Egr-1 response seen in this study. Yet it could be a response localized to the lung but not unique to DCS: similar studies involving other gas emboli or thrombus may help define the Egr-1 response.

Is the Egr-1 response specific to the lungs? It is currently not known if Egr-1 is elevated in other tissues classically affected by DCS such as major joints and the spine. Additional work is required to determine if Egr-1 is a marker for DCS in these tissues. Defining the role of this increase in Egr-1 to determine if it is in response to nitrogen insults to the lung on ascent, if it is indicative of a supra-normal pulmonary stress response, or if Egr-1 results in a cascade of events culminating in the clinical picture of DCS is a logical next step in investigating the complexity of the stress response to DCS.

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